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Dual Methylation Pathways in Lignin Biosynthesis

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Caffeoyl–coenzyme A (CoA) *O*-methyltransferase (CCoAOMT) has been proposed to be involved in an alternative methylation pathway of lignin biosynthesis. However, no direct evidence has been available to confirm that CCoAOMT is essential for lignin biosynthesis. To understand further the methylation steps in lignin biosynthesis, we used an antisense approach to alter *O*-methyltransferase (*OMT*) gene expression and investigated the consequences of this alteration. We generated transgenic tobacco plants with a substantial reduction in CCoAOMT as well as plants with a simultaneous reduction in both CCoAOMT and caffeic acid *O*-methyltransferase (CAOMT). Lignin analysis showed that the reduction in CCoAOMT alone resulted in a dramatic decrease in lignin content. The reduction in CCoAOMT also led to a dramatic alteration in lignin composition. Both guaiacyl lignin and syringyl lignin were reduced in the transgenic plants. However, guaiacyl lignin was preferentially reduced, which resulted in an increase in the S/G (syringl/guaiacyl) ratio. We have also analyzed lignin content and composition in transgenic plants having a simultaneous reduction in both CCoAOMT and CAOMT. The reduction in both OMTs resulted in a further decrease in total lignin content. This is in sharp contrast to the effect that resulted from the reduction in CAOMT alone, which only decreased the syringl lignin unit without a reduction in overall lignin content. These results unequivocally demonstrate that methylation reactions in lignin biosynthesis are catalyzed by both CCoAOMT and CAOMT.

INTRODUCTION

Lignin, a complex phenylpropanoid polymer, is the second most abundant natural product after cellulose. It is deposited mainly in cell walls of supporting and conducting tissues, such as fibers and tracheary elements. The mechanical rigidity of lignin strengthens these tissues so that tracheary elements can endure the negative pressure generated from transpiration without collapse of the tissue. The evolution of lignin deposition is considered to be one of the key events during the evolution of primitive vascular plants. In addition to providing mechanical support, lignin has significant protective functions. Both the physical toughness and chemical durability of lignin may deter feeding by herbivores. Lignification is a frequent response to infection or wounding: it may provide a physical barrier to block the penetration of pathogens (Davin and Lewis, 1992; Boudet et al., 1995; Whetten and Sederoff, 1995; Douglas, 1996).

Lignin is derived from dehydrogenative polymerization of monolignols, namely, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Based on tracer studies and subsequent enzymatic analysis of the intermediate steps, it has been shown that monolignols are synthesized through a phenylpropanoid biosynthetic pathway (Neish, 1968). Although the lignin biosynthetic pathway has been relatively well studied, there still are some unanswered questions regarding the steps involved in methoxylation. It has long been considered that caffeic acid *O*-methyltransferase (CAOMT) is the conventional *O*-methyltransferase (OMT) in lignin biosynthesis (Boudet, 1998). We have recently shown that the expression of caffeoyl–coenzyme A (CoA) *O*-methyltransferase (CCoAOMT) is closely associated with lignifying tissues in a number of dicot species, including *Zinnia*, forsythia, tobacco, alfalfa, soybean, and tomato. According to these findings, we have proposed that CCoAOMT is involved in an alternative methylation pathway of lignin biosynthesis in *Zinnia*, and that the CCoAOMT-mediated methylation pathway is likely a general one in lignin biosynthesis during normal growth and development (Ye et al., 1994; Ye and Varner, 1995; Ye, 1997).

However, this alternative methylation pathway has not been widely accepted due to lack of genetic evidence for its presence. The main challenge to the role of CCoAOMT in lignification was that a reduction in CAOMT alone in transgenic plants effectively blocked syringyl lignin production (Dwivedi et al., 1994; Atanassova et al., 1995; Doorsselaere

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et al., 1995; Tsai et al., 1998). Thus, doubts have been cast on the role of CCoAOMT in the conversion of 5-hydroxyferuloyl–CoA to sinapoyl–CoA, even though CCoAOMT may catalyze the conversion of caffeoyl–CoA to feruloyl–CoA in monolignol biosynthesis. Therefore, genetic proof of the role of CCoAOMT in lignification is essential for the unequivocal assignment of the CCoAOMT-mediated methylation pathway in monolignol biosynthesis.

CCoAOMT was first found in carrot and parsley cell cultures, and its activity in the cultured cells was shown to be rapidly induced by treatment with fungal elicitors (Matern et al., 1988; Kühnl et al., 1989; Pakusch et al., 1989). Since the first cloning of a CCoAOMT cDNA from parsley (Schmitt et al., 1991), CCoAOMT cDNAs have been isolated from a number of species, such as *Zinnia* (Ye et al., 1994), aspen (Meng and Campbell, 1995), *Stellaria longipes* (Zhang et al., 1995), *Vitis vinifera* (Busam et al., 1997), and tobacco (Martz et al., 1998).

In this study, we used an antisense approach to decrease the CCoAOMT activity in transgenic tobacco plants. We also generated transgenic plants with a simultaneous reduction in both CCoAOMT and CAOMT activities. Although a reduction in CAOMT alone only blocked syringyl lignin synthesis without a reduction in lignin content (Atanassova et al., 1995; Doorsselaere et al., 1995), a reduction in CCoAOMT dramatically altered both lignin content and composition. Combinational effects were found in transgenic plants having a simultaneous reduction in both CCoAOMT and CAOMT. The role of CCoAOMT and CAOMT in the control of monolignol synthesis is discussed.

RESULTS

Generation of Transgenic Plants with a Reduction in CCoAOMT

Four different CCoAOMT cDNAs were isolated from a cDNA library made from tobacco stems. Sequence analysis showed that the four cDNAs can be separated into two groups: CCoAOMT1 and CCoAOMT2 in group I and CCoAOMT3 and CCoAOMT9 in group II. Within the same group, they shared >94% nucleotide sequence identity in the coding region. There was 87% nucleotide sequence identity in the coding region between the two groups. To ensure a high efficiency of antisense inhibition, we decided to use one cDNA from each group (CCoAOMT1 and CCoAOMT9, respectively) to construct the cassettes for antisense expression (Figure 1). The coding regions of these CCoAOMT cDNAs were placed downstream of the constitutive cauliflower mosaic virus (CaMV) 35S promoter in an antisense orientation. Tobacco leaf discs were transformed with Agrobacterium harboring the binary vector containing the antisense cassettes, and transgenic plants were screened for a reduction in OMT activities.

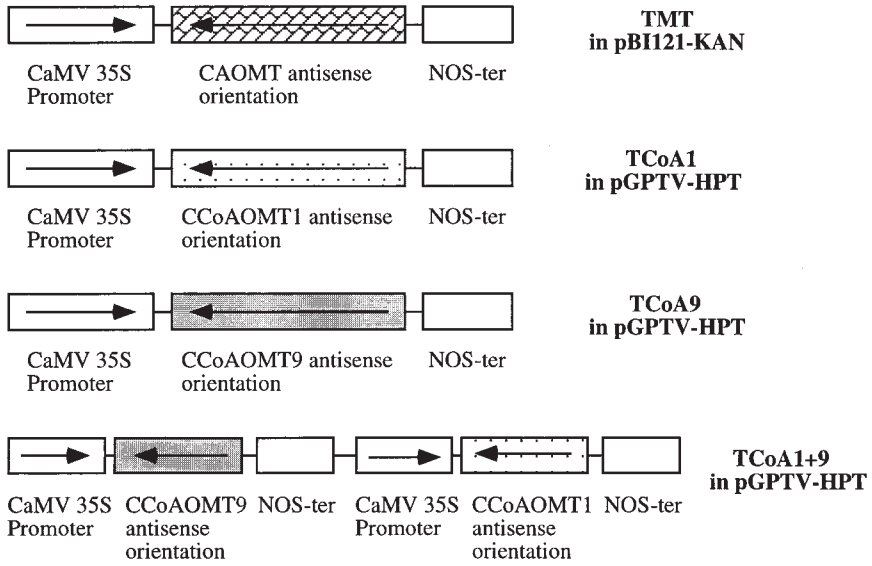

Figure 1. Diagrams of the Antisense Expression Constructs of CCoAOMT and CAOMT.

The antisense construct TMT was constructed in the binary vector pBI121-KAN, which contains a kanamycin resistance gene. The antisense constructs TCoA1, TCoA9, and TCoA1+9 were constructed in the binary vector pGPTV-HPT, which contains a hygromycin resistance gene. Antisense expression of the cDNAs is driven by the CaMV 35S promoter and terminated by the polyadenylation signal from the nopaline synthase gene (NOS-ter) of the Agrobacterium Ti plasmid.

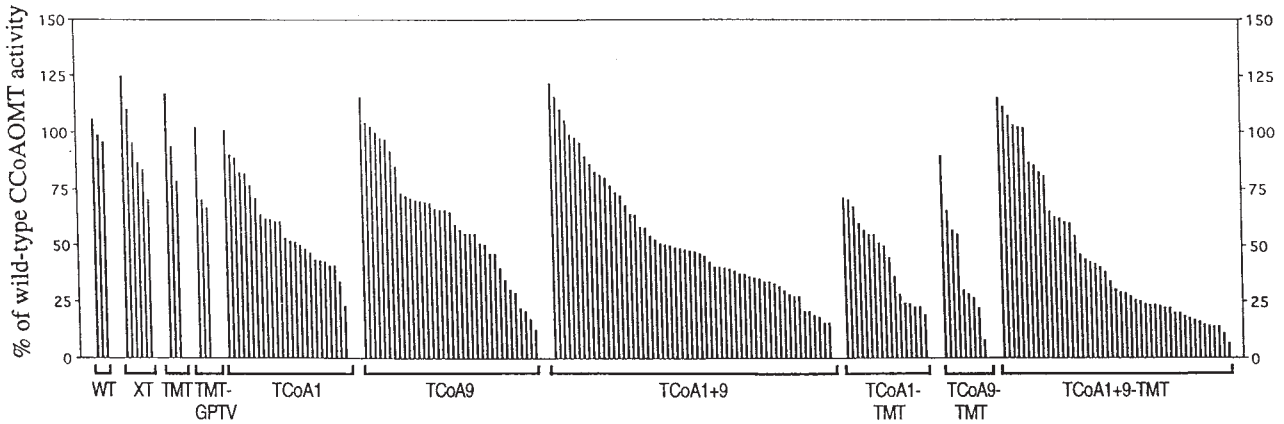

Figure 2. CCoAOMT Activity in Transgenic Tobacco Plants.

Basal stems of mature plants were used for the CCoAOMT activity assay. CCoAOMT activity in the wild-type plants was expressed as 100%. CCoAOMT activity in the transgenic plants was expressed as a percentage of wild-type activity. Each vertical line represents the value for a single plant. WT, wild type; XT, wild type transformed with the binary vector pGPTV-HPT alone; TMT, plants with antisense expression of CAOMT; TMT-GPTV, TMT plants retransformed with the pGPTV-HPT vector alone; TCoA1, plants with antisense expression of CCoAOMT1 alone; TCoA9, plants with antisense expression of CCoAOMT9 alone; TCoA1+9, plants with antisense expression of both CCoAOMT1 and CCoAOMT9; TCoA1–TMT, plants with antisense expression of both CCoAOMT1 and CAOMT; TCoA9–TMT, plants with antisense expression of both CCoAOMT9 and CAOMT; and TCoA1+9–TMT, plants with antisense expression of CCoAOMT1, CCoAOMT9, and CAOMT.

Of the 120 transgenic plants analyzed (TCoA1, TCoA9, and TCoA1+9), 43% of them showed >50% reduction in CCoAOMT activity (Figure 2). Four percent of 25 TCoA1 plants had <30% of the CCoAOMT activity found in the wild type. Seventeen percent of 37 TCoA9 plants and 24% of 58 TCoA1+9 plants had <30% of the CCoAOMT activity found in the wild type. It appears that CCoAOMT was more efficiently suppressed by the antisense expression of CCoAOMT9 than that of CCoAOMT1. Control plants transformed with the vector alone (XT) did not show a reduction in CCoAOMT. Two lines (TCoA9-164 and TCoA1+9-123) were selected for further analysis. CCoAOMT activity in TCoA9-164 and TCoA1+ 9-123 was reduced to 23 and 28% of the CCoAOMT activity found in the wild type, respectively. However, CAOMT activity was largely unaltered in these particular transgenic lines (Figure 3).

Protein gel blot analysis confirmed the activity assay results (Figure 4). The intensity of the two specific bands recognized by the antibodies raised against CCoAOMT was significantly reduced in TCoA9-164 and TCoA1+9-123 compared with that of the wild type. The proteins encoded by group II *CCoAOMT* genes were ~1 kD larger than those encoded by group I *CCoAOMT* genes. Thus, the upper band on the protein gel blot corresponded to group II CCoAOMT, and the lower band corresponded to group I CCoAOMT. Because the intensity of both bands was low in the TCoA9-164 line, this indicated that the antisense expression of CCoAOMT9 was sufficient for suppression of all CCoAOMTs. However, it appeared that the group II *CCoAOMT* genes were preferentially suppressed in the transgenic lines.

Consistent with the CAOMT activity assay, the intensity of the band recognized by the antibodies against CAOMT in TCoA9-164 and TCoA1+9-123 was the same as that of the wild type (Figure 4). These results demonstrate that expression of the CCoAOMT antisense cDNAs resulted in a specific reduction in CCoAOMT expression.

The transgenic lines were further examined for the insertion of antisense cassettes into the plant genome (Figure 5). A CCoAOMT1- or CCoAOMT9-specific primer together with a primer corresponding to the CaMV 35S promoter region were used to amplify respective sequences in the transgenic plants. The results showed that TCoA9-164 had only CCoAOMT9 antisense cDNA, and TCoA1+9-123 had both CCoAOMT1 and CCoAOMT9 antisense cDNAs, consistent with the sequence in the cassettes used for transformation (Figure 1). Genomic DNA isolated from the wild-type plants did not show any amplification with these primers.

Generation of Transgenic Plants with a Reduction in Both CCoAOMT and CAOMT

To investigate the effects of reduction in both CCoAOMT and CAOMT on lignin biosynthesis, we introduced the cassettes containing CCoAOMT and CAOMT antisense cDNAs into tobacco plants. This was done by first introducing the TMT antisense construct (Figure 1) into wild-type plants to create TMT lines and then by introducing the TCoA antisense constructs (Figure 1) into a TMT line, which had ~30% of the CAOMT activity found in the wild type. Of the

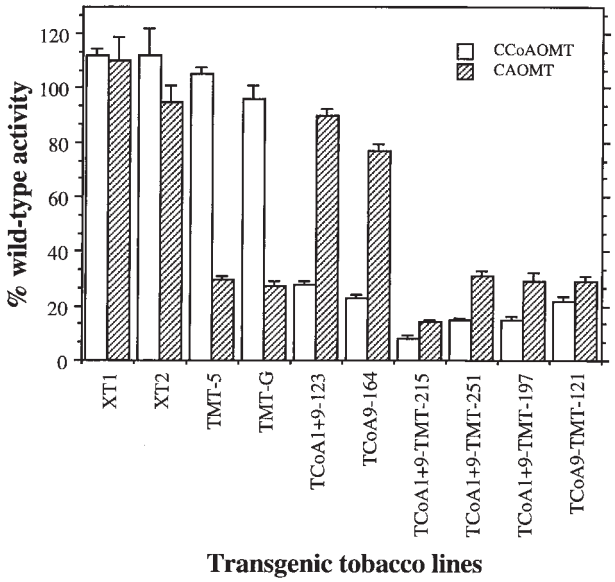

Figure 3. CCoAOMT and CAOMT Activities in Transgenic Tobacco Plants.

A few plants from each group containing antisense OMT cassettes were selected for assay of both CCoAOMT and CAOMT activities. OMT activities in transgenic plants were expressed as a percentage of wild- type activity. Both TMT and TCoA transgenic lines had a >70% reduction in CAOMT and CCoAOMT activities, respectively. Abbreviations are as given for Figure 2. Data are the mean ±SE of three assays.

73 transgenic plants analyzed (TCoA1–TMT, TCoA9–TMT, and TCoA1+9–TMT), 59% of them showed a >50% reduction in CCoAOMT activity (Figure 2). Four lines (TCoA1+9–TMT-197, TCoA1+9–TMT-215, TCoA1+9–TMT-251, and TCoA9–TMT-121) were selected for further analysis. In these transgenic lines, CCoAOMT activity was ~8 to 21% of that of the wild type, and CAOMT activity was ~14 to 31% of that of the wild type (Figure 3).

Protein gel blot analysis showed that the intensity of both CCoAOMT and CAOMT bands in the transgenic lines (TCoA1+9–TMT-197, TCoA1+9–TMT-215, and TCoA1+9–TMT-251) was significantly reduced (Figure 4). Similar to the TCoA9-164 and TCoA1+9-123 lines, the group II CCoAOMT band was almost not detectable, whereas a faint signal for the group I CCoAOMT band was still present in the transgenic lines. Interestingly, only CAOMT and group II CCoAOMT were significantly suppressed in TCoA9–TMT-121. The incorporation of the CCoAOMT and CAOMT antisense cDNAs into the plant genome was confirmed by polymerase chain reaction amplification of specific insertion sequences. Consistent with the antisense cassette sequences (Figure 1), TCoA1+9–TMT-197, TCoA1+9–TMT-215, and TCoA1+9–TMT-251 lines all had antisense sequences of CCoAOMT1, CCoAOMT9, and CAOMT. TCoA9–TMT-121 had antisense sequences of both CCoAOMT9 and CAOMT (Figure 5).

Reduction in CCoAOMT Dramatically Decreases Lignin Content and Alters Lignin Composition

To investigate whether a reduction in CCoAOMT had any effect on lignification, we collected mature stems of the transgenic lines TCoA9-164 and TCoA1+9-123 to assay for lignin content and composition. Quantitative measurement of Klason lignin (Kirk and Obst, 1988) showed that in TCoA9-164 and TCoA1+9-123, lignin content was reduced to 53 and 67% of that of the wild type, respectively (Table 1). This indicated that a reduction in CCoAOMT alone was sufficient to decrease lignin content and that the CAOMT-mediated methylation pathway could not compensate for the loss of CCoAOMT.

To examine whether the decrease in lignin content accompanied a reduction in both guaiacyl lignin and syringyl lignin, we analyzed lignin composition in both wild-type and transgenic lines. The results presented in Table 1 showed that a reduction in CCoAOMT resulted in a decrease in both guiaicyl lignin and syringyl lignin. In TCoA9-164 and TCoA1+9-123, guaiacyl lignin was reduced to ~50% of that of the wild type, and syringyl lignin was decreased to ~60% of that of the wild type. This differential decrease of guaiacyl and syringyl lignin led to an increase in the S/G (syringl/guaiacyl) ratio from ~0.75 in the wild type to ~0.95 in the transgenic lines.

Effects of a Simultaneous Reduction in Both CCoAOMT and CAOMT on Lignification

To examine the effect of a reduction in both CCoAOMT and CAOMT on lignin content and composition, we used the transgenic lines TCoA1+9–TMT-197, TCoA1+9–TMT-215,

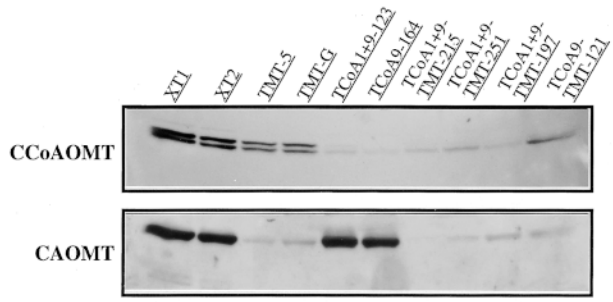

Figure 4. Protein Gel Blot Analysis of CCoAOMT and CAOMT in Transgenic Tobacco Plants.

Ten micrograms of protein extracted from stems of transgenic plants was loaded in each lane. The blot was probed with either antibodies against zinnia CCoAOMT or antibodies against zinnia CAOMT. A reduction in OMTs was evident in transgenic lines, which is consistent with the activity assay results. Two bands were seen in the CCoAOMT blot. The top band corresponds to group II CCoAOMT, and the lower band corresponds to group I CCoAOMT. Abbreviations are as given for Figure 2.

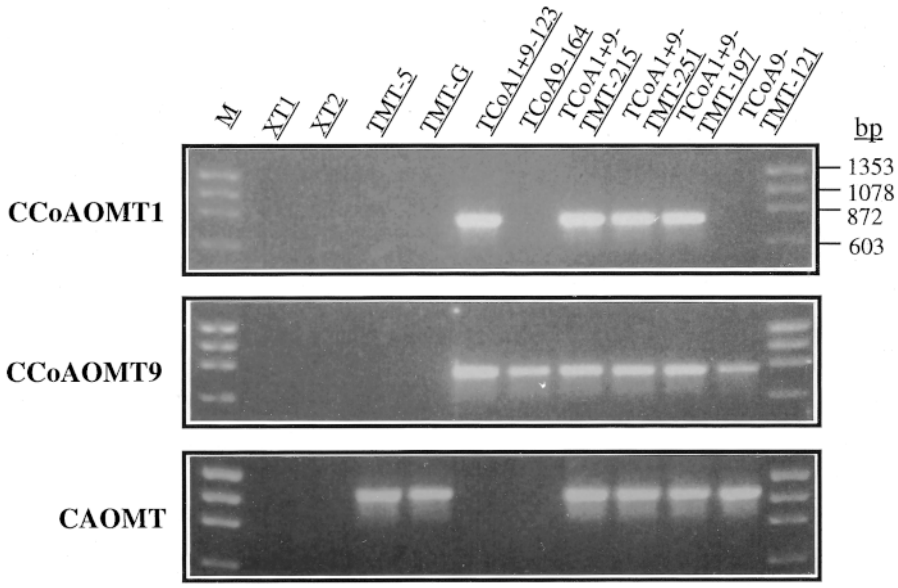

Figure 5. Presence of Antisense *OMT* Cassettes in Transgenic Plants.

Transgenic plants were examined for the insertion of antisense *OMT* cassettes into the genome. Genomic DNA from transgenic plants was used in polymerase chain reactions with a CAOMT-, CCoAOMT1-, or CCoAOMT9-specific primer together with a primer corresponding to the CaMV 35S promoter region. The results confirmed the insertion of antisense *OMT* cassettes in the plant genome. Numbers at right indicate DNA size markers (M) in base pairs. Abbreviations are as given for Figure 2.

TCoA1+9–TMT-251, and TCoA9–TMT-121 for assay of Klason lignin and lignin composition (Table 1). The Klason lignin in these transgenic lines was reduced to amounts ranging from 34 to 59% of that of the wild type. This was also revealed by pyrolysis mass spectrometry (Figure 6), a technique that has been used to evaluate a number of agricultural products (Boon, 1989). The relative intensity of mass peaks for lignin over mass peaks for cellulose, amylose, or hemicellulose was dramatically reduced in the transgenic line TCoA1+9–TMT-197 compared with that of the wild type, indicating that the lignin content relative to the cell wall polysaccharides was much lower in TCoA1+9–TMT-197 than in the wild type.

Lignin composition analysis showed that a simultaneous reduction in both CCoAOMT and CAOMT in the lines TCoA1+9–TMT-197, TCoA1+9–TMT-215, and TCoA1+9–TMT-251 had combinational effects. In addition to the decrease in guaiacyl lignin, syringyl lignin was reduced to ~15% of that of the wild type. In the line TCoA1+9–TMT-197, both guaiacyl and syringyl lignin monomers were further reduced compared with the lines with the suppression of CCoAOMT alone (TCoA9-164 and TCoA1+9-123). Guaiacyl lignin was reduced to 24% of that of the wild type in TCoA1+9–TMT-197. We do not know why TCoA9–TMT-121 showed a decrease in lignin content but no reduction in guaiacyl lignin. Because group I CCoAOMT was not reduced in TCoA9–TMT-121 (Figure 4), the group I isozyme may be sufficient to sustain synthesis of feruloyl–CoA leading to guaiacyl lignin.

Effects of Lignin Reduction on Vessel Anatomy and Plant Growth

The wild-type and transgenic plants were grown to maturity in the greenhouse. All of them grew to a similar height at a similar growth rate and flowered at similar times. The morphology of the wild-type XT-1 and transgenic plants TCoA9-164 and TCoA1+9–TMT-215 is shown in Figure 7. No visible abnormal growth was observed in the transgenic lines.

Because lignin provides mechanical strength to walls of conducting cells, we examined whether a reduction in lignin had any effect on vessel anatomy. Stem sections from both wild-type and transgenic plants were stained with phloroglucinol–HCl. In cross-section, vessel elements had a circular or an oval shape in the wild type (Figure 8) and in transgenic plants with a reduction only in CAOMT (data not shown). However, vessel walls were deformed to various degrees in transgenic plants with a reduction in CCoAOMT alone as well as in plants with a reduction in both CCoAOMT and CAOMT. The severest deformation of vessel walls was seen in the transgenic lines TCoA1+9–TMT-197, TCoA1+9–TMT-215, and TCoA1+9–TMT-251. Representative sections from TCoA9-164 and TCoA1+9–TMT-215 are shown in Figure 8.

Table 1. Lignin Content and Composition in Wild-Type and Transgenic Tobacco Plants

Transgenic Lines	Klason Lignin[a]		Lignin Composition (mg/g Cell Wall)[b]		
	% of Cell Wall[c]	% of Wild-Type Klason Lignin	Guaiacyl Lignin[c]	Syringyl Lignin[c]	S/G[d]
XT1	17.3 ± 0.9	94	17.51 ± 2.98	12.26 ± 2.03	0.70
XT2	18.1 + 0.4	100	16.98 ± 0.72	13.38 ± 0.65	0.79
TMT-5	16.3 ± 0.6	89	15.33 ± 1.12	0.89 ± 0.34	0.058
TMT-G	16.3 + 0.6	89	16.99 ± 1.10	1.99 ± 0.11	0.12
TCoA1+9-123	11.9 ± 0.2	67	8.13 ± 0.69	7.37 ± 1.2	0.91
TCoA9-164	9.7 ± 0.6	53	8.21 ± 0.52	8.64 ± 0.42	1.05
TCoA1+9–TMT-215	6.2 ± 0.2	34	8.54 ± 0.53	2.00 ± 0.08	0.23
TCoA1+9–TMT-251	7.7 ± 0.8	42	8.46 ± 0.02	1.36 ± 0.31	0.16
TCoA1+9–TMT-197	7.7 + 0.4	42	4.06 ± 0.83	2.36 ± 0.09	0.58
TCoA9–TMT-121	10.7 + 0.5	59	16.18 ± 2.14	2.47 ± 0.4	0.15

[a] Klason lignin was assayed according to Kirk and Obst (1988).
[b] Lignin monolignol composition was analyzed according to Akin et al. (1993).
[c] Each data point is the mean ±SE of two separate assays.
[d] G (guaiacyl) is the sum of vanillin, acetovanillin, and vanillic acid. S (syringl) is the sum of syringaldehyde, acetosyringaldehyde, and syringic acid.

In these plants, the majority of vessel elements in primary xylem were severely crushed. Vessel elements in secondary xylem also frequently collapsed inward.

DISCUSSION

CCoAOMT Catalyzes Methylation Reactions in Monolignol Biosynthesis

Previous results showed that CCoAOMT expression was closely associated with lignified tissues in diverse dicot species, suggesting that CCoAOMT is involved in an alternative methylation pathway in lignin biosynthesis (Ye et al., 1994; Ye and Varner, 1995; Ye, 1997). However, no direct evidence has yet been presented that CCoAOMT is essential for lignin biosynthesis.

In this study, the participation of a CCoAOMT-mediated methylation pathway in monolignol biosynthesis is unequivocally demonstrated by the antisense suppression of endogenous CCoAOMT expression. In the transgenic line having 23% of the wild-type CCoAOMT activity, Klason lignin was reduced to 53% of that of the wild type. This indicates that a reduction in CCoAOMT alone is sufficient to decrease lignin content and that the CAOMT-mediated methylation pathway could not compensate for the loss of CCoAOMT. This is in sharp contrast to the effects resulting from a reduction in CAOMT alone in transgenic plants, which did not show a significant reduction in lignin content (Dwivedi et al., 1994; Atanassova et al., 1995; Doorsselaere et al., 1995; Table 1). It seems that in transgenic plants with suppression of CAOMT, CCoAOMT efficiently compensates for the loss of CAOMT to synthesize normal levels of Klason lignin.

Both Caffeoyl–CoA and 5-Hydroxyferuloyl–CoA Are Endogenous Substrates for Methylation Catalyzed by CCoAOMT

The reduction in lignin in the antisense CCoAOMT plants accompanied a decrease in both guaiacyl lignin and syringyl lignin. This provides direct proof that endogenous CCoAOMT is involved in the methylation of both caffeoyl–CoA and 5-hydroxyferuloyl–CoA. Lignin composition analysis showed that guaiacyl lignin is preferentially decreased over syringyl lignin, which resulted in an increase in the S/G ratio in the antisense CCoAOMT plants. This could be explained by the different substrate affinities exhibited by CCoAOMT and CAOMT. CCoAOMT exhibits a higher methylation rate with caffeoyl–CoA than 5-hydroxyferuloyl–CoA (Ye et al., 1994; Busam et al., 1997). Thus, antisense suppression of CCoAOMT may have a more severe effect on guaiacyl lignin production. Likewise, CAOMT exhibits a higher methylation rate with 5-hydroxyferulic acid than with caffeic acid (Campbell and Sederoff, 1996), which may result in more efficient compensation of syringyl lignin production. The combination of these effects may lead to the differential reduction of guaiacyl lignin and syringyl lignin that was observed in the antisense CCoAOMT plants. However, CAOMT could not compensate for the loss of CCoAOMT to produce normal levels of guaiacyl lignin or syringyl lignin.

We have previously shown that the expression of CAOMT and CCoAOMT is differentially and developmentally regulated in lignified tissues of *Zinnia* (Ye and Varner, 1995). It has been proposed that the differential expression of OMTs in different tissues may contribute to lignin heterogeneity in different tissues. However, because CCoAOMT had not been conclusively shown to participate in lignification at that time, this hypothesis was not as convincing. Now that

CCoAOMT is proven to participate in lignin biosynthesis, it seems very likely that the differential expression of OMTs accounts for lignin heterogeneity observed in different tissues at different developmental stages. It will be of interest to determine whether the change of lignin composition in different tissues at different developmental stages corresponds to the differential expression patterns of OMTs.

Both CCoAOMT and CAOMT Participate in Synthesis of Guaiacyl Lignin and Syringyl Lignin

It has been clearly demonstrated that antisense suppression of CAOMT alone could lead to a loss of syringyl lignin production (Atanassova et al., 1995; Doorsselaere et al., 1995). CCoAOMT does not seem to be able to compensate for the loss of CAOMT to produce syringyl lignin, although it could effectively do so to produce a normal level of guaiacyl lignin. How can one explain these observations? Because CCoAOMT has been proven to participate in the conversion of 5-hydroxyferuloyl–CoA to sinapoyl–CoA, the logical explanation is that the putative feruloyl–CoA 5-hydroxylase does not exist in the lignified tissues (Figure 9). Thus, antisense suppression of CAOMT alone effectively reduces the availability of ferulic acid, which is the only intermediate leading to the formation of substrates for the methylation of 5-hydroxyl groups catalyzed by CAOMT or CCoAOMT. This results in effective block of syringyl lignin production. In contrast, reduction of feruloyl–CoA by the loss of CAOMT could be completely compensated by the CCoAOMT-catalyzed methylation of caffeoyl–CoA, which can be synthesized from either CoA activation of caffeic acid or hydroxylation of *p*-coumaroyl–CoA.

The lignin biosynthesis scheme shown in Figure 9 places ferulate 5-hydroxylase (F5H) in the only route leading to syringyl lignin production. This is consistent with results reported in both the mutant lacking F5H and transgenic plants overexpressing F5H. A mutation in the *F5H* gene in Arabidopsis resulted in complete loss of syringyl lignin production

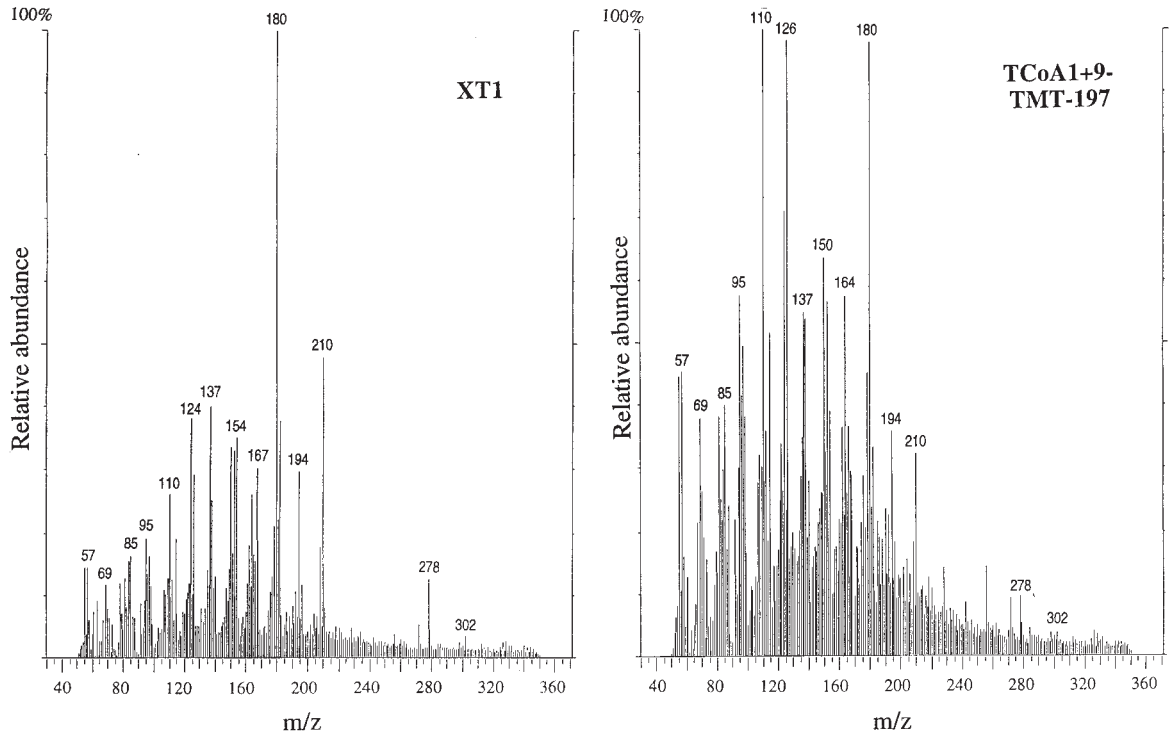

Figure 6. In-Source Pyrolysis Mass Spectrometry of Cell Walls of the Wild-Type and Transgenic Plants.

In-source pyrolysis mass spectrometry was performed using a Finnigan GCQ equipped with a direct exposure probe (rhenium loop), as described by Morrison and Archibald (1998). Mass peaks of guaiacyl lignin had *m*/*z* values of 124, 137, 138, 150, 152, 164, 166, 178, and 180. Mass peaks of syringyl lignin had *m*/*z* values of 154, 167, 168, 180, 182, 194, 196, 208, and 210. Mass peaks of cellulose and amylose had *m*/*z* values of 57, 60, 73, 85, 86, 96, 98, 100, 102, 110, 112, 126, and 144. Mass peaks of hemicellulose had *m*/*z* values of 58, 85, 86, and 114. Mass peaks for cellulose, amylose, or hemicellulose and mass peaks for lignin showed dramatic changes in relative intensity between the wild type and the transgenic line TCoA1+9–TMT-197. This is consistent with the Klason lignin assay, which showed a dramatic reduction of lignin in the transgenic line TCoA1+9–TMT-197.

Figure 7. Morphology of Wild-Type and Transgenic Plants.

Although transgenic plants had a 40 to 60% reduction in lignin content, they appeared to grow normally under the greenhouse conditions. Abbreviations are as given for Figure 2.

(Chapple et al., 1992; Meyer et al., 1996). Furthermore, overexpression of F5H in transgenic Arabidopsis plants led to predominant production of syringyl lignin, with little guaiacyl lignin accumulating (Meyer et al., 1998).

Simultaneous Reduction in Both CCoAOMT and CAOMT Results in Combinational Effects on Both Lignin Content and Composition

Although antisense suppression of CAOMT resulted in nearly complete blockage of syringyl lignin production but no reduction in guaiacyl lignin or lignin content, a decrease in CCoAOMT led to a reduction in lignin content as well as a reduction in both guaiacyl and syringyl lignin monomers. When both CAOMT and CCoAOMT were suppressed in the antisense plants, an additive effect on the reduction in lignin content and composition was observed. Simultaneous suppression of both CCoAOMT and CAOMT (TCoA1+9–TMT-197, TCoA1+9–TMT-215, and TCoA1+9–TMT-251) resulted in a further decrease in lignin content to 34 to 42% of that of the wild type compared with 53 to 67% of that of the wild type seen in the lines with antisense suppression of CCoAOMT alone (TCoA9-164 and TCoA1+9-123; Table 1). Also, syringyl lignin was further reduced to ~15% of that of the wild type in the transgenic lines with the antisense suppression of both CAOMT and CCoAOMT (TCoA1+9–TMT-197, TCoA1+9–TMT-215, TCoA1+9–TMT-251, and TCoA9–TMT-121) compared with ~60% of that of the wild type in the lines with antisense suppression of CCoAOMT alone. Because these lines showed the same amount of residual CCoAOMT, as detected by the protein gel blot analysis (Figure 4), the results are comparable.

Lignin in tobacco stems is composed of guaiacyl lignin and syringyl lignin; no significant amount of hydroxyphenyl lignin is present. Although antisense suppression of both CCoAOMT and CAOMT blocks further conversion of caffeic acid and caffeoyl–CoA, it does not appear to result in any increase in hydroxyphenyl lignin. This indicates that the absence of hydroxyphenyl lignin in wild-type tobacco is not due to the preferential influx of intermediates toward the guaiacyl or syringyl lignin production. It is most likely due to the lack of reactions leading to conversion of *p*-coumaroyl–CoA to *p*-coumaryl alcohol. It will be interesting to examine whether a decrease in OMT activities leads to an accumulation in their substrate levels.

Antisense Suppression of CCoAOMT Has No Major Negative Effect on Plant Growth but Results in Deformation of Vessel Elements

Although antisense suppression of CCoAOMT alone or of both CCoAOMT and CAOMT resulted in a 34 to 67% reduction in lignin, no significant effects on plant growth and morphology were observed when the transgenic plants were grown in the greenhouse. The transgenic plants bore normal flowers and grew to heights similar to wild-type plants. This indicates that plants could tolerate loss of significant amounts of lignin without major effects on plant growth under greenhouse conditions. It will be interesting to investigate whether the transgenic lines with reduced lignin content could survive as normally as the wild type under stress conditions.

The phenotype exhibited by the CCoAOMT antisense plants appears to be very different from the phenotypes of plants with suppression of phenylalanine ammonia–lyase (PAL; Elkind et al., 1990), overexpression of MYB-related transcription factors (Tamagnone et al., 1998), or suppression of cinnamoyl–CoA reductase (CCR; Piquemal et al., 1998). Because PAL is at the entry point leading to the phenylpropanoid pathway and the MYB-related transcription factors regulate phenylpropanoid metabolism in general, suppression of PAL or overexpression of the MYB-related transcription factors alters biosynthesis of diverse phenolic compounds that may be important for plant growth

and survival. Thus, it is expected that severe phenotypes are associated with these changes (Elkind et al., 1990; Tamagnone et al., 1998).

In contrast, the block of CCoAOMT does not affect the biosynthesis of important compounds involved in defense, such as flavonoids, chlorogenic acids, and salicylic acid. Thus, the block of CCoAOMT may be more specific to monolignol biosynthesis. The residual level of monolignols produced in the transgenic plants may be sufficient to sustain normal plant growth. Similarly, no abnormal phenotype was observed in transgenic plants with >50% reduction in thioglycolic acid–extractable lignin resulting from suppression of hydroxycinnamate:CoA ligase (Lee et al., 1997). However, this could not explain the phenotypic difference between the CCoAOMT antisense plants and CCR antisense plants. In one CCR antisense line with 47% reduction in Klason lignin, normal plant growth was dramatically affected (Piquemal et al., 1998). This implies that some factors other than the reduction in lignin content may contribute to the abnormal phenotype observed in CCR antisense plants because the CCoAOMT antisense plants with up to 66% lignin reduction grew as normally as the wild type. Further study is necessary to clarify this difference. In addition, a more thorough analysis of the impact of lignin reduction on plant growth and survival should be performed in the transgenic lines with suppression of OMTs.

Although no visible abnormal phenotype was observed in the OMT antisense plants, the vessel shape was significantly altered. This is similar to what has been observed in CCR antisense tobacco plants (Piquemal et al., 1998) as well as in bean plants grown in the presence of a PAL inhibitor, L-α-aminooxy-β-phenylpropionic acid (Amrhein et al., 1983; Smart and Amrhein, 1985). In both cases, a reduction in lignin resulted in the collapse of vessel elements. Because the mechanical rigidity of lignin strengthens vessel walls, it is obvious that the lack of lignin weakens the vessel walls so that they can no longer withstand the negative pressure generated through transpiration, thus resulting in collapse of vessel elements. It will be interesting to determine the threshold of lignin reduction at which vessel wall strength is not affected. However, it seems that even with some deformed vessel elements, plants could still transport water and solutes efficiently to support normal growth.

The demonstration of the essential roles of CCoAOMT in lignification has not only enriched our knowledge of the lignin biosynthetic pathway but also implies the potential utility of CCoAOMT as a target for genetic engineering of lignin. Because no abnormal visible phenotype was associated with substantial reduction in lignin in the OMT antisense plants, it is anticipated that CCoAOMT alone as well as the combination of both CCoAOMT and CAOMT may be ideal targets for modification of lignin content and composition in trees and forage crops. Furthermore, the preferential reduction of guaiacyl lignin over syringyl lignin, leading to a higher S/G ratio in CCoAOMT antisense plants, presents additional benefits to the pulping process because lignin with higher S/G ratios requires less chemicals for the kraft pulping process (Chiang et al., 1988).

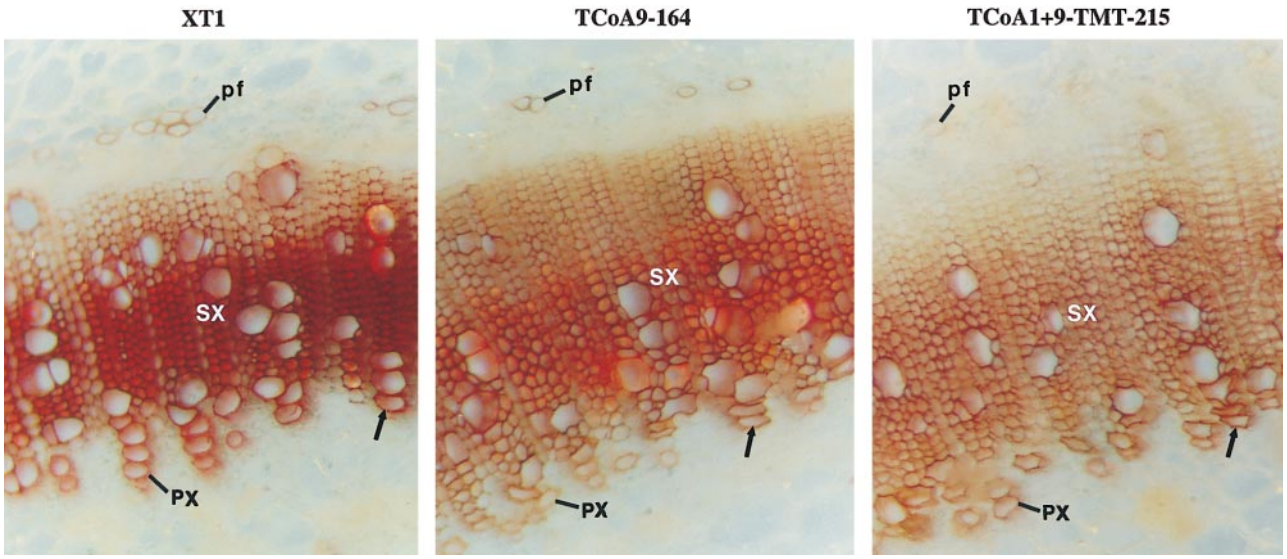

Figure 8. Vessel Anatomy in Stems of Transgenic Plants.

Stem sections were stained with phloroglucinol–HCl to show anatomy. Vessel elements (arrows) in both TCoA9-164 and TCoA1+9–TMT-215 were deformed. pf, phloem fiber; PX, primary xylem; SX, secondary xylem. Abbreviations for transgenic lines are as given in Figure 2.

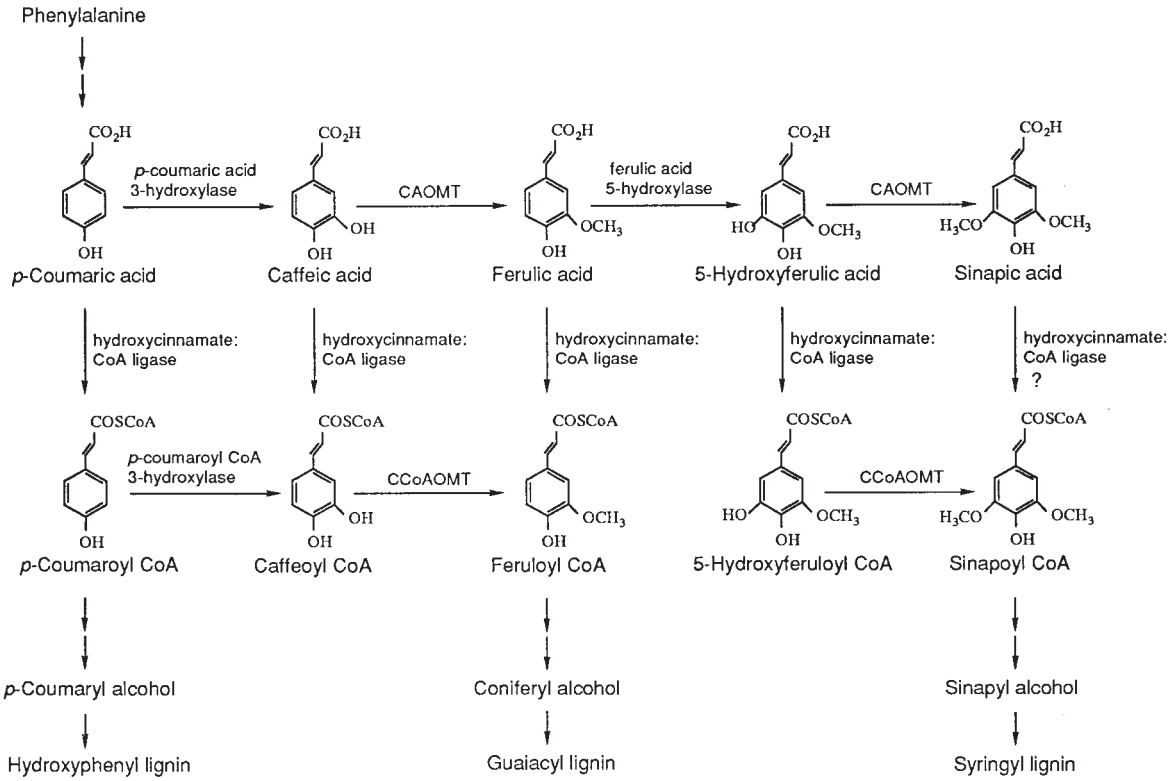

Figure 9. A Scheme of Monolignol Biosynthesis.

The methylation steps are shown in this scheme. Our findings unequivocally demonstrated that CCoAOMT participates in monolignol biosynthesis. The reduction in CCoAOMT resulted in a dramatic decrease in lignin content as well as a decrease in both guaiacyl and syringyl lignin units. Because a block of CAOMT or ferulic acid 5-hydroxylase expression led to almost complete absence of syringyl lignin units, it is logical to suggest that the conversion of feruloyl–CoA to 5-hydroxyferuloyl–CoA does not occur. The question mark indicates the reaction that may not occur due to the low activity of hydroxycinnamate:CoA ligase toward sinapic acid (Lee et al., 1997).

METHODS

Materials

Tobacco (*Nicotiana tabacum* cv Xanthi) plants were grown in the greenhouse. Caffeoyl–coenzyme A (CoA) was synthesized as described by Negrel and Smith (1984).

Isolation of Tobacco CCoAOMT and CAOMT cDNAs

Tobacco cDNAs synthesized from mRNAs isolated from tobacco stems were ligated into the cloning vector lambda ZAPII (Stratagene, La Jolla, CA) to generate a tobacco stem cDNA library. The library was used for immunoscreening of caffeoyl–CoA *O*-methyltransferase (CCoAOMT) and caffeic acid *O*-methyltransferase (CAOMT) cDNAs with antibodies against *Zinnia* CCoAOMT and *Zinnia* CAOMT, respectively, as described by Sambrook et al. (1989). Positive clones were identified and converted into phagemids. cDNAs were sequenced in both directions. Comparison of the cDNA sequences to those in the public databases was performed using the BLAST network service from the National Center for Biotechnology Information (Bethesda, MD).

Construction of Cassettes for Antisense Expression of CCoAOMT and CAOMT cDNAs

Cassettes for Antisense Expression of CCoAOMT cDNAs

To create the cassette for antisense expression of the CCoAOMT1 cDNA, we used polymerase chain reaction (PCR) with two primers (5′-TTGCTTGATATCATGGCTGAGAACGGTGCAGCAC-3′ and 5′-TTGCTTACTAGTCAG/TG/CTGATGCGGCGGCACA-3′) to amplify the CCoAOMT1 cDNA fragment. The amplified fragment was cut with SpeI and EcoRV and ligated into the SpeI and EcoRV sites of pBI221 vector (Clontech, Palo Alto, CA), creating the first shuttle construct pBI221–TCoA1AS. The CCoAOMT1 cDNA, together with the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*NOS*) terminator, was isolated from pBI221–TCoA1AS by digestion

with EcoRI, filling in with the Klenow fragment of DNA polymerase I, and then digestion with PstI. The isolated fragment was ligated into PstI and EcoRV sites of pBluescript KS+ vector (Stratagene) to create the second shuttle construct pBS–35STCoA1AS. The CCoAOMT1 cDNA together with CaMV 35S promoter and *NOS* terminator was cut out from pBS–35STCoA1AS by XhoI and SmaI and ligated into the SalI and end-filled EcoRI sites of the binary vector pGPTV-HPT (Becker et al., 1992) to create the antisense expression construct TCoA1 (Figure 1).

To create the cassette for antisense expression of CCoAOMT9 cDNA, we used PCR with two primers (5′-TTGCTTGATATCATGGCAGAGAACGGAATTAAAC-3′ and 5′-TTGCTTACTAGTCAG/TG/CTGATGCGGCGGCACA-3′) to amplify the CCoAOMT9 cDNA fragment. The same strategy used for the construction of TCoA1 was used to ligate the amplified CCoAOMT9 cDNA fragment into the binary vector pGPTV-HPT, creating the antisense expression construct TCoA9 (Figure 1).

To create the cassette for antisense expression of both CCoAOMT1 and CCoAOMT9 cDNAs, we cut out the CCoAOMT9 cDNA together with the CaMV 35S promoter and the *NOS* terminator from pBS–35STCoA9AS by digestion with SalI, filling in with the Klenow fragment of DNA polymerase I, and then digestion with XbaI. The fragment was ligated into the SmaI and XbaI sites of the plasmid pBS–35STCoA1AS. The DNA fragment containing both CCoAOMT1 and CCoAOMT9 antisense cassettes was cut out by XhoI and SmaI and ligated into the SalI and end-filled EcoRI sites of the binary vector pGPTV-HPT, creating the antisense expression construct TCoA1+9 (Figure 1).

Cassette for Antisense Expression of CAOMT cDNA

The CAOMT cDNA fragment in pBluescript plasmid was cut out by EcoRV and SacI. The isolated fragment was then ligated into the SmaI and SacI sites of the binary vector pBI121, creating the antisense expression construct TMT (Figure 1).

Tobacco Transformation and Regeneration

The constructs TMT, TCoA1, TCoA9, and TCoA1+9 were transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation. Tobacco leaf discs were transformed with Agrobacterium, as described by Horsch et al. (1985). TMT transformants were selected by growing on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 300 mg/L kanamycin and 500 mg/L carbenicillin. TCoA transformants were selected by growing on Murashige and Skoog medium containing 50 mg/L hygromycin and 500 mg/L carbenicillin. After rooting, transgenic plants were transferred to soil and grown in the greenhouse.

Preparation of Crude Extracts and Assay of Enzyme Activity

Tobacco stems were homogenized in extraction buffer (50 mM Tris-HCl, pH 7.5, 0.2 mM $MgCl_2$, 2 mM DTT, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) with a mortar and pestle. After homogenization, the extracts were centrifuged at 12,000*g* for 15 min. Supernatants were saved for both CCoAOMT and CAOMT activity assays (Pakusch et al., 1989; Ye et al., 1994).

Protein concentration was determined according to the method of Bradford (1976) with BSA as the standard protein.

Protein Gel Blot Analysis

Proteins were separated on 4 to 20% gradient SDS–polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Sambrook et al., 1989). The membranes were incubated in blocking buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 5% nonfat milk) for 4 hr and then incubated with the polyclonal antibodies against CCoAOMT or CAOMT fusion protein (1:5000 dilution) in the blocking buffer overnight. In the control experiment, the antibodies were replaced with preimmune serum. After washing, the membranes were incubated with peroxidase-conjugated goat anti–rabbit polyclonal antibodies (1:10,000 dilution in the blocking buffer) for 1 hr. The signals were detected with Amersham ECL chemiluminescent reaction reagents, according to the manufacturer's protocol.

Lignin Content and Composition Analysis

Lignin content was quantitatively measured using the Klason method (Kirk and Obst, 1988). Briefly, stems were ground into fine powder. After four extractions in methanol and vacuum drying, 200 mg of the sample was hydrolyzed in 4 mL of 72% H_2SO_4 at 30°C for 1 hr. The hydrolysate was diluted by the addition of 112 mL of H_2O and then autoclaved for 1 hr. The sample solution was filtered through a fritted glass crucible. The sample was washed and dried, and then lignin was measured and expressed as a percentage of the original weight of cell wall residues.

Lignin composition was determined as described by Akin et al. (1993) and Morrison et al. (1996). Briefly, the ethanol-extracted stem samples were hydrolyzed in 4 N NaOH at 170°C for 2 hr. The hydrolysate was acidified with 2 N HCl to pH 2.0. The released lignin monomers were extracted into diethylether and vacuum dried. The residue was dissolved in 10 μL of pyridine and 10 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and analyzed for phenolics by gas–liquid chromatography. Compounds were identified by comparison of their mass spectra with published spectra or those of the authentic compounds. All samples were run in duplicate.

In-Source Pyrolysis Mass Spectrometry

In-source pyrolysis mass spectrometry was performed on a Finnigan GCQ mass spectrometer equipped with a direct exposure probe (rhenium loop) (Thermoquest, San Jose, CA), as described by Morrison and Archibald (1998). Methanol-extracted stem samples were suspended in distilled water by using a glass mortar and pestle. A small amount of the suspension was placed on the loop, and the water was evaporated under vacuum. Analysis conditions were as follows: ionization energy of 20 eV; mass range of 50 to 500; scan time of 1 sec; temperature rise of ~10°C per second to 700°C; and ion source temperature of 175°C.

Histological Staining of Lignin

Tobacco stems were free-hand sectioned with a razor blade, and sections were stained for lignin with phloroglucinol–HCl (1%

phloroglucinol in 6 N HCl). Sections were observed under a dissection microscope by using dark-field illumination.

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